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Conjugation of Palmitic Acid Improves Potency and Longevity of siRNA Delivered via Endosomolytic Polymer Nanoparticles

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Abstract

Clinical translation of siRNA therapeutics has been limited by the inability to effectively overcome the rigorous delivery barriers associated with intracellular-acting biologics. Here, in order to address both potency and longevity of siRNA gene silencing, pH-responsive micellar nanoparticle (NP) carriers loaded with siRNA conjugated to palmitic acid (siRNA-PA) were investigated as a combined approach to improve siRNA endosomal escape and stability. Conjugation to hydrophobic PA improved NP loading efficiency relative to unmodified siRNA, enabling complete packaging of siRNA-PA at a lower polymer:siRNA ratio. PA conjugation also increased intracellular uptake of the nucleic acid cargo by 35-fold and produced a 3.1-fold increase in intracellular half-life. The higher uptake and improved retention of siRNA-PA NPs correlated to a 2- and 11-fold decrease in gene silencing IC₅₀ in comparison to siRNA NPs in fibroblasts and mesenchymal stem cells, respectively, for both the model gene luciferase and the therapeutically relevant gene PHD2. PA conjugation also significantly increased longevity of silencing activity following a single treatment, as observed in fibroblasts. Thus, conjugation of PA to siRNA paired with endosomolytic NPs is a promising approach to enhance the functional efficacy of siRNA in tissue regenerative and other applications.

Keywords

Gene knockdown; endosomolytic nanoparticle; siRNA conjugation

Introduction

Therapeutic application of RNA interference provides the potential to potently and specifically suppress transcription factors, subsets of kinases, and other signaling molecules that are traditionally considered “undruggable”. Unfortunately, achieving successful *in vivo* delivery of siRNA has proven a complex and difficult challenge, impeding medicinal translation of siRNA therapeutics. siRNA has a short half-life due to nuclease susceptibility, rapid renal clearance if injected intravenously, and an inability to translocate the membranes that make up the outer cell surface and the vesicles of the endolysosomal recycling/degradation pathways^{1–3}. Numerous and varied strategies have been developed in attempts to address these delivery obstacles, including modifications to the siRNA backbone, the use

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of lipids and polymers as carrier systems, and the conjugation of siRNA to cell-penetrating/cell-binding peptides, antibodies, dendrimers, and lipid-like molecules^{1, 4–8}. Tremendous progress has been made, especially in the application of lipid nanoparticles for delivery of siRNA against hepatic targets, but widespread medicinal application of siRNA remains a distant goal^{9, 10}. Thus, there is a continued need to better elucidate the ideal siRNA chemistry and its potential synergism with polymeric- and lipid-based carriers, with the goal of discovering combinations that will expand the therapeutic use of siRNA for a broader set of clinical indications.

Work from our lab and others has focused on block copolymers that can package and deliver siRNA to the cytoplasm, facilitated by pH-dependent membrane disruptive activity that promotes endosomal escape^{5, 11–14}. In the current work, we sought to improve the performance of siRNA formulations with these promising polymers, with the goal of improving efficacy and longevity of action such that functional effects can be achieved at a reduced polymer/siRNA dose. Lessening the dose of the carrier is anticipated to be advantageous in reducing nonspecific effects, as high doses of both cationic lipids and polymers can induce production of inflammatory cytokines and interferons, global changes in gene expression, and toxicity^{1, 6, 15, 16}.

Hydrophobization of nucleic acid delivery systems has been demonstrated to enhance carrier stability and transfection efficiency in a broadly applicable manner^{7, 12, 17, 18}. The conjugation of hydrophobic moieties to siRNA can increase siRNA nuclease resistance and enhance cellular internalization without detrimentally impacting gene silencing activity^{19, 20}. Direct conjugation of siRNA to cholesterol and α -tocopherol improves gene silencing efficacy *in vivo*, especially when the target site is the liver and after incubation of the conjugated siRNA with lipoproteins^{21–23}. Conjugation of palmitic acid (PA) to siRNA has shown particular promise as a modification strategy to improve siRNA stability, cellular penetration, and gene silencing²⁰. PA is an endogenous post-transcriptional modification commonly found on membrane-associated signaling proteins; while PA is involved in a wide variety of cellular functions, it is especially believed to influence protein-membrane interactions as well as protein uptake and intracellular trafficking^{24–26}. Motivated by the inherent effects of PA on membrane binding and translocation, PA-modified siRNA has been recently tested as an approach to enhance gene silencing in comparison to unmodified siRNA or siRNA conjugated to cholesterol in cancer cell lines *in vitro*^{19, 20}. In these studies, PA conjugation was demonstrated to enhance knockdown of siRNA delivered via Lipofectamine 2000 (a commercial transfection reagent with established cytotoxicity) and also to enable some gene silencing in the absence of a transfection reagent at high (micromolar) doses^{16, 19}.

These promising siRNA conjugate results motivated the current study, which was aimed at testing siRNA-PA conjugates for delivery via endosomolytic polymer-based nanoparticles and at assessing PA conjugates in different cell lines relevant to regenerative medicine applications. The ultimate goal of these studies is to enhance the therapeutic potency in order to minimize the quantity of polymeric carrier and siRNA required, thus reducing the potential for nonspecific effects. We have previously delivered polymeric siRNA nanoparticles from porous, biodegradable polyester urethane (PEUR) scaffolds to achieve

tunable and potent gene silencing^{11, 27}. This approach for local siRNA delivery has shown tremendous potential in applications for tissue regeneration, especially through local silencing of prolyl hydroxylase domain protein 2 (PHD2) to promote wound healing. PHD2 negatively regulates hypoxia inducible factor 1 α , a pro-angiogenic transcription factor, and scaffold-based delivery of PHD2 siRNA activates a pro-angiogenic, pro-growth program^{27–29}. The current studies were designed to assess the potential of PA conjugation for enhancing the potency and longevity of gene silencing of siRNA for ultimate application in wound healing.

Experimental Methods

Materials

Amine-modified single-stranded DNA (modification at 5' end) or Dicer substrate siRNA (modification at 3' end) and complementary single-stranded Cy5-modified DNA or unmodified Dicer substrate siRNA were obtained from Integrated DNA Technologies (Coralville, Iowa). The pGreenFire1-CMV plasmid was obtained from System Biosciences (Mountain View, CA), and packaging plasmids pMDLg/pRRE, pRSV-Rev, and pMD2.G were purchased from Addgene (Cambridge, MA). Lipofectamine 2000 and NucBlue Fixed Cell ReadyProbes were purchased from Life Technologies (Grand Island, NY). RNEasy spin columns were obtained from Qiagen (Venlo, Netherlands), and the iScript cDNA Synthesis Kit from Bio-Rad (Hercules, CA). NIH-3T3s and HEK-293Ts were obtained from ATCC (Manassas, VA), and mouse marrow stromal cells (C57Bl/6-TgNs) were purchased from Jackson Laboratory (Bar Harbor, ME). Propylacrylic acid was synthesized as previously reported^{1,2}. CytoTox-ONE Homogeneous Membrane Integrity Assay (a lactate dehydrogenase (LDH) assay) was purchased from Promega (Madison, WI). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Oligonucleotide-PA Synthesis and Characterization

Single-stranded amine-modified oligo was reacted with 100-fold molar excess of PA *N*-hydroxysuccinimide ester pre-dissolved at 40 mM in *N,N*-dimethylformamide (DMF). The reaction was carried out for 18 hours at room temperature in 45% water, 45% isopropyl alcohol, and 10% DMF. The oligo-PA was purified by reversed-phase HPLC using a Clarity Oligo-RP column (Phenomenex, Torrance, CA) under a linear gradient from 95% water (50 mM triethylammonium acetate), 5% methanol to 100% methanol. The conjugate molecular weight was confirmed using MALDI-TOF mass spectrometry (Voyager-DE STR Workstation, Grand Island, NY) using 50 mg/mL 3-hydroxypicolinic acid in 50% water, 50% acetonitrile with 5 mg/mL ammonium citrate as a matrix. The yield of the oligo-PA was quantified based on absorbance at 260 nm. The purified oligo-PA was annealed to its complementary strand to generate Cy5-modified DNA-PA or siRNA-PA. Conjugation and annealing was also confirmed via agarose gel electrophoresis.

Oligonucleotide-loaded Nanoparticle (NP) Synthesis and Characterization

A diblock copolymer composed of a homopolymer of 2-(dimethylamino) ethyl methacrylate (DMAEMA) blocked with a random copolymer of DMAEMA, 2-propylacrylic acid (PAA), and butyl methacrylate (BMA) was synthesized using reversible addition-fragmentation

chain transfer (RAFT) polymerization as described previously^{5, 11}. Assembly of NPs was triggered by dissolving polymer in 100% ethanol, followed by slow addition of PBS or water via syringe pump. siRNA or DNA (with or without PA) was mixed with NPs and allowed to electrostatically condense for 30 minutes. Dynamic light scattering (DLS; Zetasizer Nano-ZS Malvern Instruments Ltd., Worcestershire, U.K.) and transmission electron microscopy (TEM; FEI Tecnai Osiris, Hillsboro, OR) were used to analyze size and zeta potential of oligo-loaded NPs. siRNA and siRNA-PA NPs were imaged by TEM. DLS was performed on NPs loaded with DNA, DNA-PA, siRNA, or siRNA-PA. Gel electrophoresis was used to test loading efficiency at varied amine:phosphate (N:P) ratios. A red blood cell hemolysis assay was used to determine pH-dependent membrane disruptive activity of oligo NPs as a marker for endosomal escape functionality.³⁰

Production of Stable Luciferase-Expressing Cell Lines

To produce lentivirus, the pGreenFire1-CMV plasmid and packaging plasmids pMDLg/pRRE, pRSV-Rev, and pMD2.G were transfected into HEK-293Ts using Lipofectamine 2000. Media was changed after 24 hours and supernatant containing lentivirus was collected at 48 and 72 hours. Viral supernatant was added directly to NIH-3T3s with 6 µg/mL polybrene or mMSCs with 60 µg/mL protamine sulfate. Media was changed after 24 hours. Lentiviral transduction efficiency was determined by GFP expression as analyzed by flow cytometry (BD LSR II Flow Cytometer, San Jose, CA).

Luciferase Silencing

NIH-3T3s or mMSCs were treated with siRNA NPs or siRNA-PA NPs; the siRNA was either designed against the luciferase gene (luc siRNA) or was a scrambled sequence (scr siRNA). Cells were treated in 10% serum for 24 hours before imaging with an IVIS 200 imaging system (Caliper Life Sciences, Hopkinton, Massachusetts). Unless otherwise noted, an N:P ratio of 4:1 was used. To evaluate treatment cytotoxicity, media was removed and cells were washed gently with PBS and lysed. An LDH measure was used to determine cell number, with a decrease in cell number corresponding to cell death during treatment. In the investigation on longevity of luciferase silencing, the media was changed after 12 hours of treatment to 2% serum to prevent cellular overgrowth. Growth in low serum was used to reduce the confounding influence of proliferation and allowed more direct investigation of changes in gene silencing longevity due to increased intracellular stability.

PHD2 Gene Silencing

NIH-3T3s were treated with siRNA NPs or siRNA-PA NPs at an N:P ratio of 4:1 as described above except for using prolyl-hydroxylase 2 (PHD2) siRNA. Cells were treated in 10% serum for 24 hours and then incubated for 24 hours, and the degree of PHD2 knockdown was quantified by real time polymerase chain reaction (RT-PCR) using the Ct method and normalizing to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Cellular Uptake and Retention

DNA was used as a simple model for siRNA in uptake studies as justified by similar physicochemical properties of the analogous NPs (Figure S1). NIH-3T3s were treated with

Cy5-labeled DNA NPs or DNA-PA NPs in 10% serum for 12 hours at 12.5 nM. To measure intracellular retention with minimal dilution effects due to cell proliferation, media was then changed to 2% serum, and cells were cultured for up to an additional 72 hours. Intracellular fluorescence was quantified using flow cytometry at 12, 36, 60, and 84 hours. Extracellular membrane-bound fluorescence was quenched with Trypan Blue. For confocal microscopy imaging studies, NIH-3T3s were treated with 25 nM Cy5-labeled DNA NPs or DNA-PA NPs in 10% serum for 12 hours before media was changed to 2% serum. Again, growth in low serum was used to reduce the confounding influence of proliferation and to enable more direct investigation of intracellular persistence. Cell nuclei were stained with DAPI (using NucBlue Fixed Cell ReadyProbes), and cellular fluorescence was imaged using confocal microscopy at 12, 36, and 60 hours.

To assess the mechanism of uptake of PA-conjugated oligo NPs, NIH-3T3s were pre-treated for 30 minutes with either Dynasore (30 μ M), 5-(N-ethyl-N-isopropyl) amiloride (EIPA) (30 μ M), or cytochalasin D (5 μ M), which are inhibitors of clathrin/caveolin-mediated endocytosis, macropinocytosis, and caveolin-mediated endocytosis/macropinocytosis, respectively. Cy5-labeled DNA-PA NPs were then added at 50 nM, and the cells were incubated with both the inhibitors and NPs for 4 hours in 10% serum. Intracellular fluorescence was then quantified using flow cytometry with extracellular fluorescence quenched with Trypan Blue.

Statistical Methods

The levels of luminescence or gene expression of the treatment groups were statistically compared using a One Way ANOVA test coupled with a Tukey means comparison test, with a p-value of less than 0.05 deemed representative of a significant difference between groups. In all cases, the arithmetic mean and standard deviation are reported/plotted and the sample size (n) is indicated.

Results

Synthesis and Characterization of Oligo-PA NPs

PA-conjugated single-stranded oligos were successfully purified from the reactants via HPLC, and the desired products were confirmed by MALDI-TOF analysis (Figure 1) and also by an upward shift of the free PA-siRNA band in comparison to the unmodified siRNA band on an agarose gel (Figure 2). Unmodified siRNA showed full loading into the diblock polymer NPs at an N:P ratio of 6:1, while PA-modified siRNA was fully loaded at a ratio of 4:1 (Figure 2). siRNA-PA NPs were ~8 nm larger than unmodified siRNA NPs (49 nm vs. 41 nm), as demonstrated by DLS and TEM (Figure 3). Zeta potential was equivalent between siRNA-PA NPs and unmodified siRNA NPs. To validate the use of DNA/DNA-PA NPs as a cost-effective model for siRNA/siRNA-PA NPs, the size and zeta potential of all four NP formulations were compared (Figure S1). No change in size distribution or zeta potential was observed between DNA/siRNA NPs and DNA-PA/siRNA-PA NPs. No difference in pH-dependent membrane disruptive activity (i.e., as a measure of endosomolytic behavior) was detected between unmodified oligo NPs and oligo-PA NPs (Figure S2).

Gene Silencing Efficacy of siRNA-PA NPs

The influence of the difference in loading efficiency (i.e., N:P ratio required for full packaging) between unmodified and PA-modified siRNA was first investigated for gene silencing. Superior silencing was detected for luc-PA NPs in comparison to luc NPs at all N:P ratios tested (Figure 4). The silencing activity of luc NPs was low at N:P ratios less than 4:1, and there was a significant increase in silencing when the N:P was raised from 4:1 to 6:1. However, there was significant gene silencing at all ratios tested, even an N:P of 1:1, for luc-PA NPs, and the benefit of adding extra polymer saturated at an N:P of 4:1. Based on previous precedent and the observation that mild but statistically significant cytotoxicity was observed at N:P of 6:1 (Figures S3 and S4), a 4:1 ratio was utilized for subsequent comparative studies⁵. Observation of cytotoxicity at 6:1, as evaluated by luciferase measurements on scrambled controls (Figure S3) and the LDH assay (Figure S4), emphasizes the potential impact of achieving more efficient loading and activity with a smaller quantity of polymeric carrier.

At a fixed N:P ratio of 4:1, increased gene silencing bioactivity of luc-PA NPs over luc NPs was apparent in both mouse fibroblasts (NIH-3T3s) and mMSCs (Figure 5). The IC₅₀ was calculated based on equation 1 where y is the fraction luciferase activity, x is the concentration in nM, and b is a fit parameter.

$$y = \frac{-1}{(1 + (\frac{x}{IC_{50}})^b)} + 1 \quad \text{Equation 1}$$

The IC₅₀ of luc-PA NPs was 2.3-fold and 11.0-fold lower than that of luc NPs in the fibroblasts and the mMSCs, respectively, indicating that siRNA conjugation to PA significantly improves the gene silencing potency at a 4:1 N:P ratio. Importantly, no significant difference in luminescence was seen in cells treated with the scr NPs compared to the scr-PA NPs (a reduction in luminescence after treatment with the scrambled controls would indicate non-specific effects or cytotoxicity) (Figure S5). As evaluated by the luciferase activity in the cells treated with scrambled control siRNA, no significant cytotoxicity was seen below a 40 nM dose of siRNA NPs in the fibroblasts or at any of the siRNA NP doses tested in the mMSCs. No cytotoxicity was detected for any siRNA-PA NP doses tested in the fibroblasts or mMSCs.

The impact of PA modification on efficiency of silencing of the therapeutically-relevant gene PHD2 was also investigated. PHD2-PA NPs significantly enhanced gene silencing in comparison to PHD2 NPs when evaluated by RT-PCR (Figure 6). In fibroblasts, the IC₅₀ of PHD2-PA NPs was 2.3-fold lower than that of PHD2 NPs. These data agree well with the data acquired using luc siRNA, confirming that the bioactivity gained through PA conjugation is consistent across different siRNAs.

Cellular Uptake and Retention of Oligo-PA NPs

To mechanistically explore the improved bioactivity achieved through PA conjugation, the cellular uptake and retention of fluorescently modified DNA NPs or DNA-PA NPs was evaluated by flow cytometry at an N:P of 4:1. The intracellular fluorescence of fibroblasts

treated with DNA-PA NPs was 35-fold higher than those treated with the same dose of DNA NPs (Figure 7A). Because free fluorophore molecules not attached to nucleic acids are rapidly exocytosed (within minutes), intracellular fluorescence was used as a marker of intracellular persistence of the intact molecule³¹. The intracellular half-life of the DNA vs. the DNA-PA was investigated by monitoring the intracellular fluorescence over time after treatment removal and fitting the data to equation 2 where y is the fluorescence, y_0 the initial fluorescence measured at the time of treatment removal, x is the time in hours, and λ is related to the half-life $t_{1/2}$ by equation 3. Using these equations, the intracellular half-life of DNA-PA was found to be 3.1-fold higher than that of unmodified DNA.

$$y = y_0(e^{-\lambda x}) \quad \text{Equation 2}$$

$$t_{1/2} = \frac{\ln(2)}{\lambda} \quad \text{Equation 3}$$

In support of the flow data, confocal microscopy revealed visibly increased fluorescence of fibroblasts treated with DNA-PA NPs in comparison to DNA NPs (Figure 7B). The overexposure of the images depicting DNA-PA NP uptake was necessary to demonstrate the uptake of DNA NPs under the same imaging settings and results from the large difference in uptake of the fluorescently labeled siRNA between the two treatments. Additionally, microscopy images at 0, 24, and 48 hours post-removal of treatment supported the flow cytometry results, demonstrating better retention of PA conjugates over time. Importantly, untreated cells showed no fluorescence when imaged using the same microscope settings (Figure S6).

Next, uptake pathway inhibitors were used to investigate the uptake mechanism of DNA-PA NPs in fibroblasts. EIPA, an amiloride analog and inhibitor of macropinocytosis, and cytochalasin D, which disrupts actin filament formation and thus impedes both caveolae-mediated uptake and macropinocytosis, inhibited uptake of the DNA-PA NPs by >65%. Treatment with Dynasore, which blocks a dynamin GTPase necessary for formation of all clathrin-coated vesicles and for uptake through caveolae, resulted in a 40% decrease in uptake of DNA-PA NPs (Figure 8). A parallel uptake inhibition study was conducted using unmodified DNA NPs; for this study, there was a statistically insignificant 30% inhibition of uptake in cells treated with Dynasore and no significant uptake inhibition for samples treated with Cytochalasin D or EIPA. However, the lower baseline level of uptake of the DNA NPs may have reduced the sensitivity to measure their uptake inhibition.

Longevity of Gene Silencing of siRNA-PA NPs

The longevity of gene silencing of luc-PA NPs in comparison to unmodified luc NPs was investigated in NIH-3T3 fibroblasts. The duration of luciferase silencing with luc-PA NPs was increased in comparison to luc NPs. In cells treated by luc NPs, luciferase activity returned to equivalent to that of untreated cells by 72 hours, while cells treated with luc-PA NPs exhibited no decrease in silencing between 48 and 72 hours (Figure 9). After 7 days, cells treated with luc-PA NPs still showed 40% of the silencing effect detected at 24 hours

(28% overall luciferase silencing at 7 days). This functional longevity of action corresponds to the increased intracellular half-life of oligo-PA compared to unmodified oligo.

Discussion

Direct conjugation of siRNA to targeting ligands, lipophilic moieties, or carrier molecules is a promising approach to enhancing knockdown efficacy and/or reducing the need for additional transfection reagents^{23, 32, 33}. Palmitic acid has shown particular promise for increasing resistance to nuclease degradation, cellular uptake, and gene silencing in initial *in vitro* tests using commercial lipid-based reagents²⁰. Herein, we have characterized the performance of siRNA-PA relative to unmodified siRNA delivered via endosomolytic, polymer-based NPs that have previously demonstrated efficacy both *in vitro* and for local scaffold-based delivery *in vivo*^{5, 27}. The goal was to improve the potency and longevity of action of the siRNA and to reduce the potential for negative side effects in ultimate scaffold-based siRNA-NP delivery applications.

The siRNA-PA was successfully synthesized using a one-step reaction and purified using HPLC (Figure 1). An important advantage of siRNA-PA over unmodified siRNA was its improved NP packaging. siRNA-PA completely loaded into the NPs at a 4:1 N:P ratio, while unmodified siRNA necessitated a 6:1 ratio for the polymer utilized in these studies (Figure 2). These data suggest that hydrophobization of siRNA is an efficient means to improve siRNA cargo loading and stability of NPs¹⁸. Other reports that nanoparticle carriers loaded with lipid-modified siRNA form particles at lower N:P ratios and with increased stability also support this conclusion^{32, 34, 35}. It is posited that interactions among siRNA molecules through the lipophilic moieties increase negative charge density and improve loading by cationic agents and that aggregated lipophilic moieties also contribute to micelle stability^{32, 35, 36}. Another potential contributing factor, in our NP carriers in particular, is that the PA molecule may help to anchor the siRNA into the hydrophobic micellar nanoparticle core. These hydrophobic interactions could help to reinforce the electrostatic interactions between the siRNA and the poly(DMAEMA)-based NP corona. This hypothesis is supported by the finding that siRNA-PA NPs had larger hydrodynamic diameter than siRNA NPs (Figure 3), while unloaded NPs and siRNA NPs were the same size (Figure S1). These data suggest that siRNA-PA impacts the overall NP packing, potentially due to increased interaction with the NP core.

Next, siRNA-PA NP silencing of both the model gene luciferase and of the therapeutically relevant gene PHD2 was benchmarked against siRNA NPs in fibroblasts. Similar studies were also done in MSCs, which have relevance for tissue regenerative applications and represent a cell type that is notoriously challenging to transfect^{8, 37}. The first study assessed the effects of the N:P ratio used in the NP formulation on luciferase silencing in fibroblasts. At every N:P ratio tested for an siRNA dose of 40 nM, siRNA-PA NPs produced significantly greater gene silencing than unmodified siRNA NPs (Figure 4). The largest improvement in silencing by siRNA-PA occurred at the lower N:P ratios of 1:1, 2:1, and 4:1. Beyond a ratio of 4:1, increasing the amount of polymer had no significant impact on target knockdown for siRNA-PA NPs. Achieving potent silencing with a lower N:P is key to minimizing detrimental treatment effects, as a charge ratio of 6:1 caused measureable

cytotoxicity in fibroblasts (Figure S3, S4). The siRNA-PA NPs achieved a high level (more than 70%) of silencing efficacy even at an N:P of 1:1, which was superior to unmodified siRNA loaded at a 4:1 N:P ratio (the standard formulation condition for this polymeric NP composition)^{5, 11}.

Gene silencing potency was also compared for the siRNA-PA NPs and unmodified siRNA NPs formed at a constant N:P of 4:1. In fibroblasts, the dose of siRNA-PA NPs required to achieve 50% gene silencing was consistently two- to three-fold lower than the dose of siRNA NPs needed to achieve this effect. The improved potency of siRNA-PA NPs was apparent both for the model gene luciferase and for the therapeutically-relevant gene PHD2 (Figure 5A, 6). An even greater increase in gene silencing potency of 11-fold was observed in MSCs (Figure 5B). The polymer NPs used here have been shown to be promising for siRNA delivery to MSCs, while commercial reagents like Lipofectamine and Dharmafect have substantial cytotoxicity and low transfection efficiencies^{13, 25}. Our results suggest that there is a synergistic effect between this polymeric carrier and siRNA-PA for transfection of MSCs, which reinforces findings that PA modification of polymeric carriers enhanced MSC plasmid transfection²⁵. Unlike strategies to increase bioactivity by increasing the polymer:siRNA (N:P) ratio, the siRNA conjugated to PA did not have any impact on cell viability. Because the siRNA-PA conjugation decreases the amount of polymer needed and also increases the relative potency of the siRNA, it has the potential to strongly impact the therapeutic index for polymeric NP siRNA formulations.

In order to better understand the mechanism for the improved gene silencing performance of siRNA-PA NPs, cellular uptake and intracellular retention were measured. Flow cytometry revealed a 35-fold increase in cellular uptake of DNA-PA NPs relative to DNA NPs in fibroblasts. This effect was hypothesized to be due to the presence of PA moieties, which may hydrophobically interact with cell membranes. Endogenous PA modification of proteins facilitates membrane binding and endocytotic processes, and the number of lipophilic modifications on nucleic acids directly correlates to the level of association with cell membranes^{24, 26, 36}. Therefore, the presence of multiple PA moieties on the corona of the NPs may act cooperatively to facilitate uptake in combination with the positive zeta potential of the NPs. Previous studies of PA-modified siRNA employing Lipofectamine 2000 as a delivery agent showed more modest improvements in cellular uptake relative to the gains achieved herein with polymeric NPs¹⁹. This discrepancy is likely attributable to differences in the mechanism of siRNA complexation or cellular internalization achieved with each type of reagent. For example, cholesterol-modified siRNA loaded into cationic polymer micelles also demonstrated moderate cellular uptake enhancement over unmodified siRNA in comparison to the improvement achieved by siRNA-PA in our system³⁴. However, in this system, lipophilic siRNA was packaged in the core of the micelles, whereas our NP carriers load siRNA/siRNA-PA onto the micelleplex corona, potentially promoting multivalent PA interactions with the cell membrane. Because PA modification has also been shown to improve siRNA nuclease resistance, we also assessed intracellular persistence¹⁹. Indeed, not only the level of uptake, but also the intracellular retention was improved by PA conjugation for siRNA delivered via endosomolytic polymer nanoparticles (Figure 7).

The improved level of uptake and intracellular persistence achieved through PA conjugation motivated subsequent examination of the cellular uptake mechanism of oligo-PA NPs. Investigation into the uptake mechanism of PA-conjugated oligo NPs revealed a larger role for macropinocytotic uptake in comparison to clathrin and caveolin-mediated endocytosis, as seen by the significantly higher uptake inhibition by EIPA and cytochalasin D compared to Dynasore (Figure 8). Conversely, the level of uptake of unmodified oligo NPs was not significantly affected by EIPA and Cytochalasin D, suggesting that the increased uptake of PA-modified oligos may be due to their preferential uptake through a macropinocytic pathway. The predominance of a macropinocytotic mechanism could be due to increased hydrophobic interactions with the cell surface and/or the slightly larger size of the siRNA-PA NPs. Internalization via this pathway may also impact siRNA-PA NP intracellular retention and cytoplasmic bioavailability, as it is accepted that the endocytotic mechanism has a significant impact on the trafficking and ultimate intracellular fate of internalized therapeutics^{38, 39}. For example, macropinosomes are believed to be leakier and to provide an easier access point into the cellular cytoplasm³⁹, which may enhance the efficiency of endosomal escape and intracellular bioavailability of the siRNA-PA NPs.

We next examined whether the primarily macropinocytotic uptake mechanism, combined with the increased intracellular half-life, impacted the longevity of gene silencing. As predicted, these factors improved the duration of gene silencing (Figure 9), and taken together these data suggest significantly greater persistence and longevity of action for siRNA-PA NPs relative to siRNA NPs. This effect is anticipated to be impactful for regenerative applications where repeat administration of siRNA may not be convenient or feasible.

In conclusion, these studies indicated that siRNA conjugation to PA acts synergistically with endosomolytic polymer NP formulations. PA conjugation reduced the required ratio of polymer:siRNA and improved silencing potency, which was relevant across multiple genes and cell types relevant to tissue regeneration. Ultimately, the siRNA-PA would significantly reduce the ratio of polymer:siRNA as well as the overall dose of siRNA necessary for functional gene silencing. Furthermore, PA conjugation significantly improved longevity of silencing of siRNA. Thus, utilization of siRNA-PA NPs would facilitate a sustained functional effect with less frequent and/or lower doses, effectively broadening the therapeutic index and enhancing the probability of success for siRNA applications in tissue engineering.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Figure 2.

Figure 3.

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Figure 7.

Figure 8.

Figure 9.